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Evaluation Of Controlling Silkworm Bacterial Diseases Using Propolis Extract And Cinnamon Oil

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ABASTRACT

Silkworm (*Bombyx mori.*), It is an important economic insect which is a producer of silk. Flacherie is a pattern associated with bacterial diseases on silkworm. The incidence of diseases at the time of silkworm rearing severely reduces the production of silk. This study was designed to examine the possibility of controlling the bacterial diseases in silkworm using the ethanolic extracts of propolis and cinnamon oil extract. The infected larvae were collected and the pathogenic bacteria were isolated and identified. Antibacterial activity of two extracts was tested in Muller Hinton agar plates with the concentrations of 0, 20, 50, and 100 µl. The sensitivity rate was much effective in ethanolic extract of propolis than cinnamon oil extract in vivo and in vitro. Therefore, It can be used for controlling bacterial disease (flacherie) and improve larvae health, its weight and cocoon weight which lead to increase the silk production.

Keywords: Bacterial diseases, Silkworm, Propolis, Cinnamon Oil.

INTRODUCTION

Sericulture means cultivation of silkworms which finally produces SILK. Silk is considered to be the queen of textile and the naturally produced animal fiber (Welford, 1969 and Shelagh, 2004).

Silkworm diseases are the most important disease that inflects heavy loss to crops. Among silkworm diseases, bacterial diseases are common. Pathogenic bacteria of silkworm belong to a wide variety of genera, including *Bacillus, Enterobacter, Serratia, Aeromonas, Streptococcus, Pseudomonas and Staphylococcus* (Tao *et al*., 2011). However, when silkworms are physiologically weak, bacterial diseases can attack them, eliciting a heavy toll on sericulture (Aruga, 1994). The bacterial diseases affecting silkworm are called flacherie because the cadavers of silkworms that have died of these diseases lose elasticity, soften, and rot.

The increasing resistance of microorganisms to conventional chemicals and drugs has prompted scientists to search for novel sources of biocides with broad-spectrum activities (Abad *et al*., 2007). Since ancient times, plants and their derivatives, such as essential oils (EOs), have been used in folk medicine. Weerakkody *et al*., (2010) indicated that the clove, cinnamon, oregano, rosemary and dill are considered as the most common spices and herbs with strong antimicrobial activity. They added their essential oils containing chemical compounds such as carvacrol, cinnamaldhyde, eugenol and camphor which are identified as the major chemical components responsible for exerting antimicrobial activity.

Propolis has attracted public interest since it is a natural product with many biological properties. It was used in folk medicine as early as 300 BC for cosmetic purposes, its anti-inflammatory properties, and wound healing. It has been used internally and externally, and is believed to possess anti-bacterial, anti-viral, fungicidal, local anesthetic, anti-ulcer, anti-inflammatory, immunostimulant, hypotensive, and cytostatic properties (Banskota *et al.*, 2001). More than 300 propolis constituents have been identified by gas chromatography-mass spectrometry (GC-MS). These compounds can be grouped as follows: free aromatic acid; flavonoids; benzyle;methylbutenyl; cinnamyl and other esters of these acids; chalcones and dihydrochalcones; terpenoids and others as sugars; ketones; and alcohols (Bankova, 2005). Although in small quantities, these compounds can have important positive and negative effects on the therapeutic properties.

Few investigators studied the effect of some bee products as propolis or bee venom to controlling silkworm diseases (El-Massarawy, 1995 and Nour *et al.*, 1997). The present work was designed to control bacterial silkworm diseases by using propolis and cinnamon extracts.

MATERIALS AND METHODS

This work was carried out during the spring season of 2015 in Departments of Microbiology and in the laboratory of Sericulture of Plant Protection Department in Faculty of Agriculture, Masoura University.

This work was carried out in many several experiments:

Collection of infected larvae

Infected larvae were collected in the spring rearing at 2014. These larvae were showed bacterial infection, silkworms were soft and rot. The infected silkworm larvae were brought to the laboratory in sterilized conditions for further studies.

Source of propolis and cinnamon:

Propolis was collected from the bee hive. The propolis was cleaned and become free from wax, paint, wood etc. Then 30 g of the sample was dissolved in 83.4 mL of 70% ethanol. . Cinnamon oil was obtained from Cap. Pharm. Company.

Isolation of bacteria

The samples were surface sterilized with 70% alcohol and washed twice with distilled water. Then sterilized needle was used to pierce the body wall and withdraw body fluid. The body fluid was mixed with sterile 0.1% peptone water then homogenized. Serial dilution were carried out, then inoculated onto nutrient agar, Maconkey agar and mannitol salt agar and incubated at 37 °C for 24 h. Distinct colonies were subsequently isolated for further processing (Nataraju *et al*., 2005 and Ganesh *et al*., 2013). The isolates were coded as silkworm (SW) next to the serial number.

Identification of bacterial isolates

The isolates were purified then morphological, biochemical characterization tests were carried out to identify the bacterial isolates (Dong and Cai 2001). Finally, the isolates were identified according to Bergeys Manual of Systematic Bacteriology (Holt, 2005).

Pathogenesis of bacterial isolates

The fourth instar silkwarm were orally inoculated with bacterial suspension (2x 10^6 CFU/ml) to test the pathogenesis of the respective bacteria. The disease symptoms and mortility in silkwarm was observed (Singh *et al*., 2011).

Antibacterial activity of ethanolic extract of propolis (EEP) and cinnamon oil

The antibacterial activities of these extracts on bacterial isolates were evaluated by agar well diffusion method. One ml of cell suspension (2x10^6 CFU/ml) was introduced into dissolved and cooled to 45°C Muller-Hinton Agar medium, after the medium hardened, four wells (6mm in diameter) were made, and 0, 20, 50 and 100 µl of two extracts were poured into the wells. Petri dishes were incubated at 37°C for 24h. The diameter of inhibition zone was measured.

Minimum inhibitory concentration (MIC) assay

Minimum Inhibitory Concentrations (MICs) were determined by agar dilution test using the EEP (30%) with final concentration in the medium (0, 0.1, 0.2, 0.4, 0.8, 1.75, , 3.5, 7, 14.0) µl/ml. Agar dishes were inoculated with (2x10^6 CFU/ml) of each bacterial isolate and incubated at 37 °C for 24h. In agar dilution tests, MIC is the lowest EEP concentration where no colony growth is observed at the end of the incubation period (Burt, 2004). All experiments were performed in duplicate.

Silkworm rearing technique

Rearing of silkworm was carried out in laboratory under the hygro-thermic conditions 28±1°C and 75±5% RH, according to the technique of (Krishnaswami ,1978). Larvae were fed four times daily on mulberry leaves .The larval bed was cleaned daily. Cleaning net was used for removing the remained dried food and feces.

Larvae under investigation were divided into two groups. Each group was divided into three subgroups. One group fed on mulberry leaves treated with EEP concentrations and the other group fed on mulberry leaves supplemented with cinnamon oil concentrations using three replicates (50 larvae) for each concentration.

Mulberry leaves were dipped in the concentrations of both extracts for 5 min and left to dry then offered to larvae. Larvae were treated with disinfectants four times in both the 4th and 5th larval instars, all feeds/day at the first day and the middle of both instars. The control group of leaves was treated only with distilled water. The mature larvae were mounted in plastic collapsible montages separately treatment and replication wise and cocoon harvesting was done.

Statistical analysis

The results were compared according to Duncan multiple range test by SAS (2001) (version 9.1, SAS Institute, Cary, NC, USA).

RESULTS

Six different colonies were isolated and identified using morphological and biochemical tests which presented in Table 1. Depending on morphological and biochemical characteristics, bacterial isolates were identified as follow: SW1 *Bacillus cereus*, SW2 *Bacillus megaterium*, SW3 *Bacillus subtilus*, SW4 *Echerichia coli*, SW5 *Serratia marcescens* and SW6 *Staphylococcus aureus*. The bacterial isolates were found to be individually pathogenic to silkworm.

Different concentrations (0, 20, 50 and 100 µl) of EEP and Cinnamon oil were found to be active. In addition, the results showed that by increasing the volume of EEP and cinnamon, the antibacterial activity was increase. The data showed that the EEP is comparatively more effective than cinnamon oil. The strongest bactericidal effect was observed at concentration 100 µl followed by 50µl and 20 µl. Antimicrobial activity was observed to be higher against gram positive bacteria than gram negative bacteria.

Based on the wells diffusion results; EEP was selected for further studies. The minimum inhibitory concentrations of EEP was determined, the concentrations ranged from 0.1 to 14 µl/ml, and the results are illustrated in Fig (2). The results revealed that EEP with 7 µl /ml and 14 µl /l showed the minimum inhibitory concentrations against the Gram positive bacteria and Gram negative bacteria respectively. Also data showed that Gram positive bacteria susceptible to low EEP concentrations and Gram negative bacteria was inhibited in higher EEP concentrations.

EEP and cinnamon oil extract showed antibacterial activity against bacteria that isolated from the infected silkworm. These antibacterial agents were used to investigate the effect of controlling and feeding silkworm larvae mulberry with them. The obtained results were as follow:

Table (2) shows the effect of different concentration of antibacterial extracts on some parameters of silkworm .This results show that the second concentration of propolis is more effective than other concentration on different parameters of larvae silkworm Fig (3).

The second concentration of EEP increase the weight of larvae in initial , final instar 5^{th} and weight of cocoon .In addition, that cinnamon oil is effective in comparable with control in the second concentration Tab (2). EEP is highly significant in increasing weight of larvae and cocoon. Moreover, results in Table (2) indicated that EEP2 give the highest weight of 5^{th} larvae in initial and final 24.03, 25.53 respectively. EEP2 leads to increase the development rate of weight of larvae in initial and final 5^{th} larvae. Table (2) shows EEP2 and cinnamon2 are heavier weight of cocoon than others treatments. EEP2 is the shortest duration larvae. It takes eight days comparable others treatment 9, 9,10, 11 EEP1,cinn1,cinn2 and control respectively in Fig (3).

DISCUSSION

In the present study the bacterial isolates were identified as follow; *Bacillus cereus, Bacillus megaterium, Bacillus subtilus, Echerichia coli, serratia marcescens* and *staphylococcus aureus*. The obtained results were supported by (Abou El- Ela *et al*., 2015) who isolated bacterial strains from the infected larvae and identified them as follow: *Aeromonas sp*., *Paenibacillus macerans* (*Bacillus macerans*), *Bacillus megaterium*, *Bacillus licheniformis* and *Bacillus circulans*. (Sakthivel *et al*., 2012) who also isolated six bacterial species from infected silkworm and identified as follow; *Bacillus subtilis, Streptococcus pneumoniae, Staphylococcus aureus, E. coli, Pseudomonas fluorescence, Bacillus cereus and Klebsiella cloacae*.

Our results demonstrated that the bacterial isolates were pathogenic for silkworm, but the degree of pathogenicity varies with the type of bacterial species. Similar results were obtained by(Guo- Ping and Xi- Jie 2011) who reported that *S. marcescens* and *Bacillus* sp. had pathogenic effect on silkworm. Furthermore, (Chairman *et al*., 2012) showed that the silkworm was died after injection of the following pathogenic bacteria; *Staphylococcus aureus, Pseudomonas aeruginosa, Vibrio cholera, E. coli, Serratia marcescens* and *Bacillus thuringiensis*.

As regard the antibacterial activity of EEP and cinnamon oil extract, the results are in agreement with (Sampath *et al*. 2015) who found that among eight essential oils, lavender and cinnamon oils showed high antibacterial activity against silkworm bacterial strains. Also, (Khalil 2006) reported that the strong antibacterial activity of EEP may be attributed to the synergistic activity between its various compounds, mainly phenolics and flavonoids.

Antibacterial activity of EEP and cinnamon oil extract was observed to be higher against gram positive bacteria than gram negative bacteria because, (Vaars *et al*. 1992) Gram negative bacteria possess outer membrane surrounding the cell wall which restricts diffusion of hydrophobic compounds through its lipopolysaccharides covering. The obtained results are in agreement with those of (Rahman *et al.* 2010) who observed a marked antibacterial activity of EEP against Gram positive bacteria and limited activity against Gram negative bacteria. Moreover, the antibacterial effect of propolis may be attributed to its effect on bacterial cytoplasmic membrane and its ability to inhibit the enzyme activity, cell division and protein synthesis (Mirzoeva *et al*., 1997).

Propolis extract showed anabolic effect on silkworm (Cizmark and Metal, 1978, El- Massarawy, 1995 and Gad, 2006). Also, this work is agreed with anther results that EEP has attractive effect on biological parameters on larvae .It may be improve the health status of the larvae. In Addation this results were similar with (El-Massarawy, 1995) and Gad, 2006) who found that treatment with propolis extract yielded heavier cocoons and cocoon shells. Also increased the percentage of silk content and increased the number of deposited eggs/ female than those obtained from control.

Therefore a few studies which used propolis extract as antibacterial agent for bacterial silkworm diseases *in vivo.* According to (Thirumalaisamy *et al*. 2009) who reported that If the diseases are controlled below the economic threshold level then there will be an increase of 25 per cent silk production.

Therefore the present results are important for using the extract of propolis in controlling bacterial silkworm diseases and feeding larvae to increase weight of larva, cocoon yield and silk production.

Table (1) Morphological, cultural and biochemical characters of bacterial isolates.

Bacterial isolates	SW1	SW2	SW3	SW4	SW5	SW6
Colony properties	Irregular, cream, sticky	Circular, brown, sticky	Irregular, white, sticky	circular, smooth	White, sticky	Circular, yellow
Cell shape	Rod in chains	Rod	Rod, single or chains	Short rod	Short rod- single	Cocci- arrangement in clusters
Oxygen requirement	Aerobic	Aerobic	Aerobic	Facultative anaerobic	Facultative anaerobic	Aerobic
Gram stain	+	+	+	-	-	+
Spore formation	-	+	+	-	-	-
Motility	+	+	+	+	+	-
V.P. test	+	-	+	-	+	-
M.R. test	-	+	-	+	-	-
Indole production	-	-	-	+	-	-
Utilization of citrate	+	+	+	-	+	+
Urease test	+	-	+	-	-	+
Catalase test	+	+	+	+	+	+
Hydrolysis of gelatin	+	+	+	-	+	-
Hydrolysis of starch	+	+	+	-	-	+
Lipid hydrolysis	+	+	+	-	+	+
Casein test	+	+	+	-	+	-
Hydrogen sulphite	-		-	-	-	-
Glucose	+	+	+	+	+	+
Lactose	-	+	-	+	-	+
Sucrose	+	+	+	+	+	-
Mannitol	+	+	-	+	+	+

Table (2): Effect of different concentrations of EEP and cinnamon oil on some biological parameters of *B. mori*.

Extracts / Parameters	Initial Weight of 5th larvae(gm)	Final Weight of 5th larvae (gm)	Increasing of development rate (T/C) Initia larvae	final larvae	Cocoon Weight (gm)
Cinnamon 1	20.23^{bc}	19.90^{c}	1.14	0.9	9.37^{c}
Cinnamon 2	20.83^{b}	21.70^{b}	1.17	1	10.47^{b}
EEP1	18.47^{cd}	17.77^{d}	1.04	0.8	9.77^{bc}
EEP2	24.03^{a}	25.53^{a}	1.35	1.17	12.53^{a}
Control	17.80^{d}	21.80^{b}	1	1	9.47^{c}

***Means at each column followed by the same letter are not significantly different at 0.05. T/C: Treat / control.**

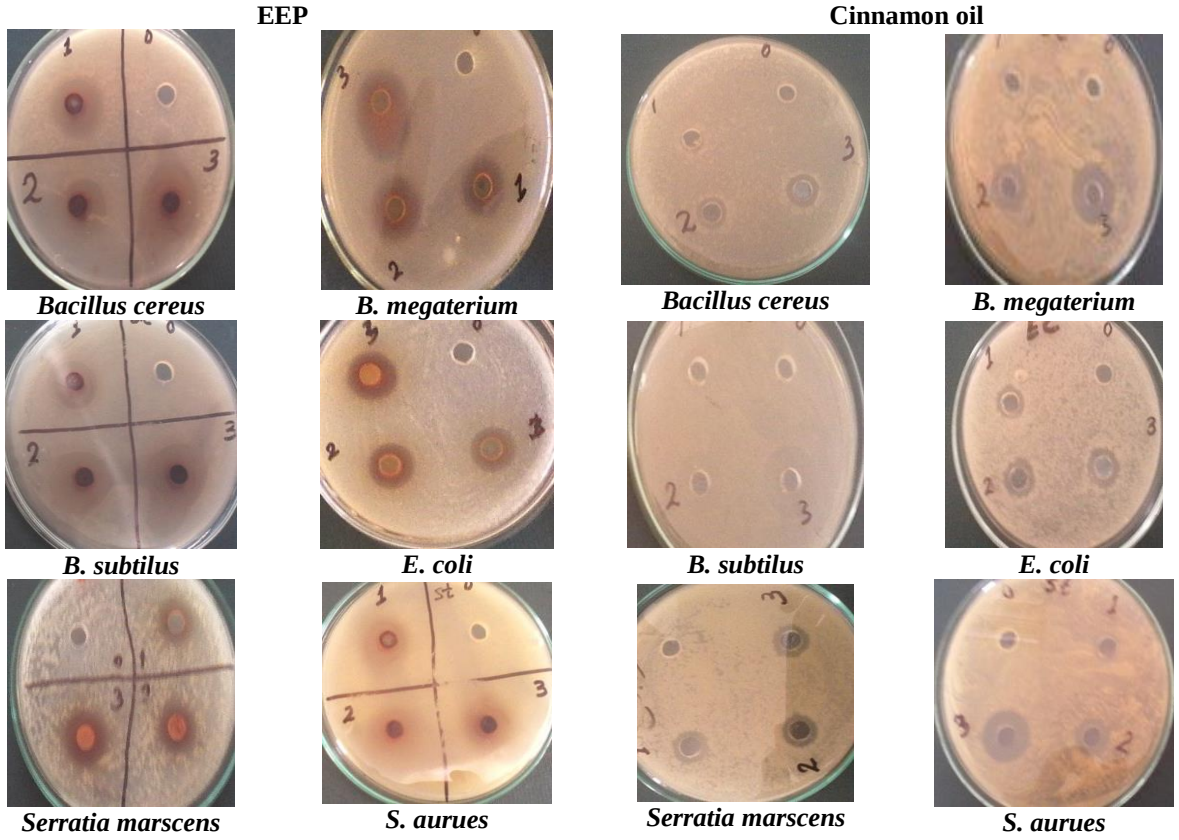

Fig (1): Effect of different concentrations of EEP and cinnamon oil on silkworm pathogenic bacteria (0 is zero, 1 is 20, 2 is 50, 3 is 100 µl of EEP and Cinnamon oil)

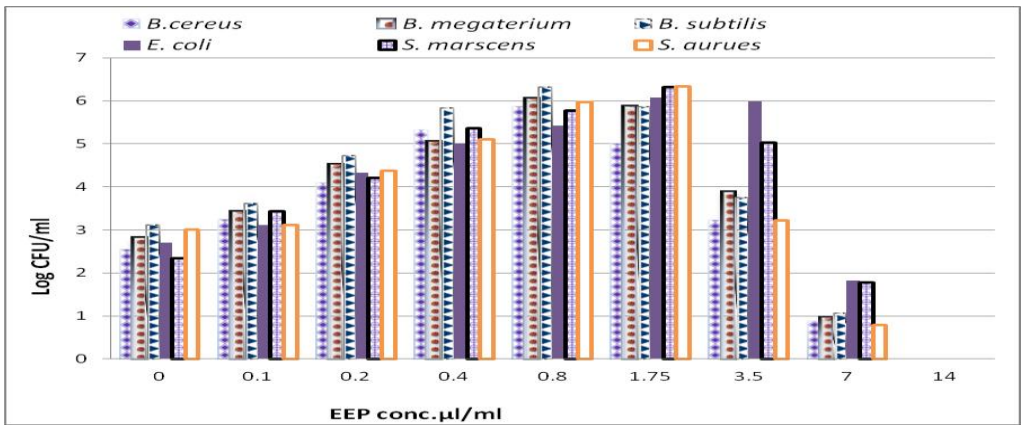

Fig (2): The minimum inhibitory concentrations of EEP

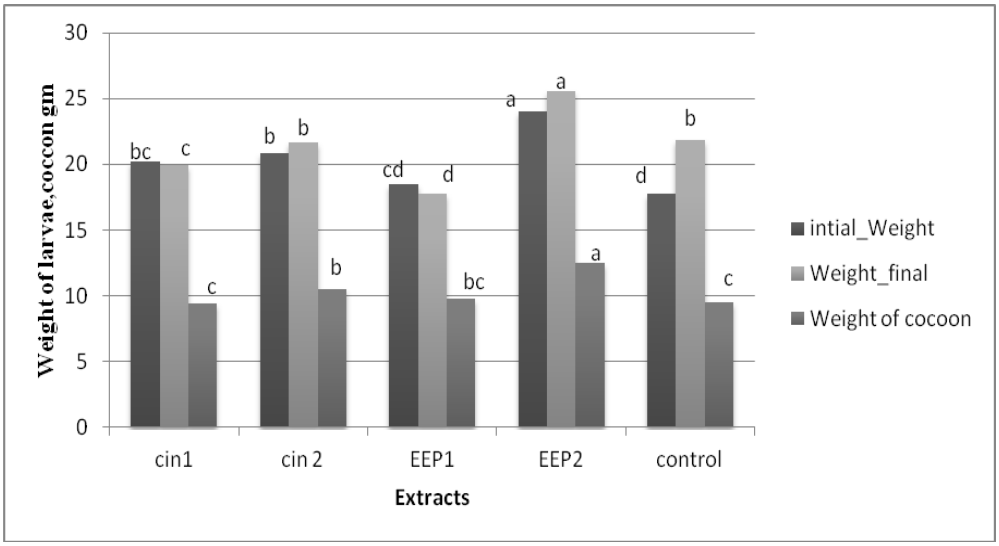

Fig (3): Effect of different concentrations of EEP and cinnamon oil on weight of larvae and cocoon.

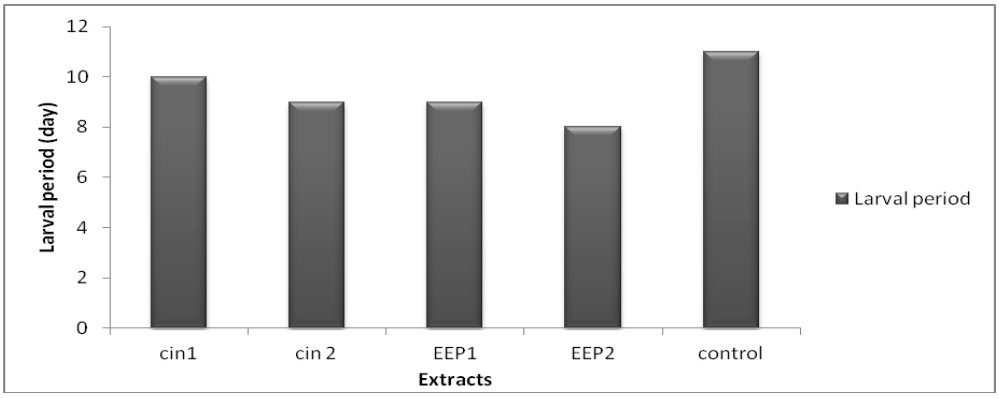

Fig (4): the larval duration (days of larval) on different concentrations of EEP and cinnamon oil

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تقييم مكافحة الامراض البكترية لديدان الحريرالتوتيه باستخدام مستخلصي البروبليس والقرفة .

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تهدف هذه الدراسة الي تقييم مكافحة الامراض البكترية لديدان الحريرالتوتية باستخدام مستخلصي البروبليس الايثانولي والقرفة وقد أ جري هذا البحث من خلال تجربتين احدهما في المختبر في قسم الميكروبيولجي والاخري في معمل التربية لديدان الحرير في كلية الزراعة –جامعة المنصورة .تم تعريف المسببات المرضية البكترية المسببة لامراض ديدان الحرير من خلال عدد من الاختبارات الحيوية والبيوكيميائية واختبار سمية هذه المسببات المرضية لها وكذلك اختبار مستخلص لكلا من البروبليس والقرفة بتركيزات مختلفة . وقد تم اجراء التجربة الثانية في معمل التربية حيث تمت التربية بطريقة الصواني علي ورق البارافين .وبناءا علي نتائج المختبروالتي اظهرت ايجابية مستخلص البروبليس الايثانولي في التركيزين (50،20 ميكروليتر/مليلتر) اكثر من مستخلص القرفة . وتمت معاملة يرقات ديدان الحرير بالتركيزين الاكثر تاثيرلكلا من المستخلصين البروبليس والقرفة. وبينت النتائج فعالية التركيز الثاني لمستخلص البروبليس الايثانولي اكثر من كل التركيزات الاخري المستخدمة وذلك من خلال وزن اليرقات في اول واخر العمر اليرقي الاخير ، وزن الشرانق وكذلك معدل الزيادة في النمو .